

In the Specification:

Page 5, delete the paragraph beginning on line 3 and substitute the following:

--The work described herein is based on a premise. The premise is that the observed instability of ribonuclease inhibitor molecules occurs because of oxidation of cysteine residues to form disulfide bridges. The thesis is further that such disulfide bridges are most likely to form when cysteine residues containing unpaired thiol groups are closely adjacent to each other. Therefore, in accordance with the method described herein, the amino acid sequence of a ribonuclease inhibitor molecule is re-designed so as to avoid having cysteine residues which are adjacent or closely adjacent in the engineered ribonuclease inhibitor. It has been found that by making this change to the amino acid sequence of ribonuclease inhibitor, and thus forming mutant ribonuclease inhibitors, those mutant ribonuclease inhibitors are more oxidation resistant and have a greater stability during handling than the wild-type ribonuclease inhibitor on which they are based. In this way, the use of ribonuclease inhibitors in biological processes becomes more practical, as the ribonuclease inhibitors become more stable and require less special treatment in order to remain active. --

Page 5, the paragraph beginning on page 5 and continuing on to page 6 (last amended by the applicant in October, 2002), delete the paragraph as previously amended and substitute the following:

--An illustration of the three dimensional structure of the human ribonuclease inhibitor is illustrated in Fig. 1. The sequence of the ribonuclease inhibitor can be found in Lee et al. Biochemistry 27:8545-8553 (1988), the disclosure of which is hereby incorporated by reference. From both Figure 1 and the sequence of the protein, it can be readily seen that some of the cysteine residues are located adjacent to each other. The amino acid residues at positions numbered 94 and 95 and 328 and 329 in the human RI sequence as numbered in Lee et al., and as illustrated in Fig. 1, are all cysteines. It was theorized that these cysteine residues would be the most likely to be oxidized to form disulfide bonds which would interfere with the biological activity of the molecule. Note that in SEQ ID NO:3 below, these cysteine residues appear as amino acids 95, 96, 329 and 330, the difference being the N-terminal methionine which is counted as residue 1 in the deduced sequence of SEQ ID: NO:3 below and as residue 0 in the sequence of Lee et al. ~~To remain consistent with prior work in the field, the numbering convention used by Lee et al. is used in this specification.~~ --

Page 7, delete the paragraph beginning on line 9, and substitute the following:

--In native human ribonuclease inhibitor, the two pairs of cysteine residues which lie most adjacent to each other are the cysteines at amino acids 94 95 and 95 96 (which are in a loop) and the cysteines at amino acid 328 329 and 239 330 which reside in an α -helix. None of these four cysteine residues are in contact with angiogenin in the complex which forms between human ribonuclease inhibitor and angiogenin. Porcine ribonuclease inhibitor has one pair of adjacent cysteine residues, which are homologous to the cysteines at residues 328 329 and 329 330 in the human RI sequence. In contrast, ribonuclease inhibitor from rat has no pairs of adjacent cysteine residues. The oxidative stability of the rat ribonuclease inhibitor protein, as well as its three-dimensional structure, is currently not known.--

Page 7, delete the paragraph beginning on line 23, and substitute the following:

--As will be discussed with the experimental results below, it was found possible to inhibit the formation of disulfide bonds between adjacent cysteine residues of a ribonuclease inhibitor by replacing the adjacent cysteine residues with alanine residues. The mutant human pancreatic ribonuclease inhibitor molecules thus created, have pairs of alanine-for-cysteine substitutions at both amino acids 94 95 and 95 96, at both amino acid positions 238 329 and 239 330, or substitutions for all four of the cysteine residues. It was demonstrated that the replacing of any or all of the cysteine residues with alanine did not markedly impair the ability of the human ribonuclease inhibitor to bind RNase A. There was, however, some slight diminution in affinity to ribonuclease for some of the variants. --

Page 7, delete the paragraph beginning on line 37, and continuing on to page 8, and substitute the following:

--It was discovered, however, that replacing these adjacent cysteine residues with alanine residues made the human ribonuclease inhibitor significantly more oxidation resistant as compared to the wild-type protein. Oxidation resistance was tested using hydrogen peroxide based on ease of laboratory use. It was discovered that the wild-type human ribonuclease inhibitor loses 50% of its activity in a solution which has little as 0.007% hydrogen peroxide volume-per-volume. By contrast, the mutant ribonuclease inhibitor having alanine substitutions at amino acid positions 328 329 and 329 330 retain 50% of its ribonuclease inhibitor activity at 0.09% volume-per-volume hydrogen peroxide. By this measure, the mutant ~~C328/C329A~~ C329/C330A ribonuclease inhibitor variant is 10 to 15 fold more resistant to oxidative damage

than is the wild-type human ribonuclease inhibitor. --

Page 9, delete the paragraph beginning on line 4, and substitute the following:

--The methodology disclosed here will be equally effective for ribonuclease inhibitor molecules from other species. Shown in Fig. 7 is a comparison of the amino acid sequences of RNASE inhibitor from rat, pig, and human. Note that pig RI shares the adjacent cysteine residues (at positions 323 and 324) corresponding to residues 328 329 and 329 330 of the human sequence, and thus could be modified as described here. The technique described here will work with all such RI molecules that natively have adjacent cysteine residues. Some RI variants, like the rat molecule illustrated in Fig. 7, contain no adjacent cysteines. --

Page 9, delete the paragraph beginning on line 16, and substitute the following:

--The goal of the work described below was to create mutant forms of human ribonuclease inhibitor which would hinder the cataclysmic oxidation of human ribonuclease inhibitor. Reasoning that the formation of disulfide bonds amongst cysteine residues in the human ribonuclease inhibitor molecule would be most likely to occur among those residues which were closest in space, it was decided to survey the three-dimensional structure of ribonuclease inhibitor to determine those residues which were closest to each other in the normal three-dimensional conformational structure of human ribonuclease inhibitor. Figure 1 is an illustration of the 3D model that was used for the structure of human ribonuclease inhibitor. Study of that structure revealed that the most proximal cysteine residues in native human ribonuclease inhibitor are those which are adjacent in the primary amino acid sequence as published by Lee et al. (*Biochemistry* 27:8545-8553 (1988)). The close amino acid residues were the cysteines at amino acid positions 94 95 and 95 96, which are in a loop structure, and the cysteines at residues 328 329 and 329 330, which are part of an alpha helix structure. None of these four cysteine residues contacts angiogenin during the formation of the ribonuclease inhibitor complex with angiogenin. It was observed that porcine ribonuclease inhibitor varies from the human sequence in that it has only one pair of adjacent cysteine residues, which are homologous to cysteines 328 329 and 329 330 in the human ribonuclease inhibitor complex. By contrast, rat ribonuclease inhibitor has no pairs of adjacent cysteine residues, but the oxidative stability of the rat protein, as well as its three-dimensional structure, is currently unknown. --

Page 11, delete the paragraph beginning on line 8, and substitute the following:

-- RNASE A for use in this work was produced in *Escherichia coli* with a recombinant DNA expression system, as described in delCardayre et al., Protein Engng. 8:261-273 (1995). Wild-type hRI and its variants were produced in *E. coli* by using plasmid pET-RI, which directs the expression of hRI as described in Leland et al., Proc. Natl. Acad. Sci. USA 95:10407-10412 (1998). To produce hRI variants, the cDNA that codes for hRI was mutated by the method of Kunkel et al. Methods Enzymol. 154:367-382 (1987). The oligonucleotides used were BMK14 (~~C94A/C95A~~ C95A/C96A; HindIII):GGCCCCCGTCAGCGCCGCGTTCTGGAGGCTAAGCTTCTG; BMK16 (~~C328A/C329A~~ C329A/C330A; NheI):GCTGAAGTGGCTAGCGGCGGCGGCTGTGAA; BMK17(~~C328A~~ C329A; SphI):GCTGAAGTGGGAGCATGCGGCGGCTGTGAA; and BMK18(~~C329A~~ C330A; NheI):GCTGAAGTGGCTAGCGCAGGCGGCTGTGAA. In these sequences, the reverse complement of new alanine codons is in bold type and new restriction endonuclease sites are underlined. cDNA sequences of mutated plasmids were determined with an ABI 373 Automated Sequencer. --

Page 13, delete the paragraph beginning on line 12, and substitute the following:

--It has been previously reported that the cysteines at residues ~~94, 95, 328, and 329~~ 95, 96, 329, and 330 of hRI do not contact angiogenin in the complex formed between human ribonuclease inhibitor and angiogenin. Thus it was not anticipated that replacing any of these cysteine residues with alanine would significantly impair the ability of human ribonuclease inhibitor to bind to ribonuclease A. Shown in Figures 3 and 4 is a graphical representation of the data showing the ability of the various modified or mutant human ribonuclease inhibitors to inhibit ribonucleolytic activity. These results demonstrate that none of the substitutions significantly impair the ability of the mutant forms of human ribonuclease inhibitor to bind to ribonuclease A. However, ~~C94A/C95A~~ C95A/C96A hRI (human ribonuclease inhibitor with cysteines at ~~94~~ 95 and ~~95~~ 96 substitute by alanine) and ~~C94A/C95A/C328A/C329A~~ C95A/C96A/C329A/C330A hRI are slightly less effective inhibitors of ribonuclease activity than is the variant ~~C328A/C329A~~ C329A/C330A. The affinity of the two single amino acid mutations variants, ~~C328A~~ C329A hRI and ~~C329A~~ C330A hRI for human ribonuclease inhibitor is between that of the wild-type human ribonuclease inhibitor and the ~~C328A/C329A~~ C329A/C330A variant as shown in Figure 4. --

Page 13, delete the paragraph beginning on line 34 (and continuing onto page 14), and substitute the following:

--The test for oxidation resistance demonstrated that replacing adjacent cysteine residues with alanine makes the resulting mutant hRI oxidation resistant. As the oxidant in this test we chose H₂O₂, which is easier to dispense than O₂ gas and which likewise oxidizes thiols to disulfides. As shown in Figure 5, H₂O₂ has a greater effect on ~~C328A/C329A~~ C329A/C330A human ribonuclease inhibitor than it has on the ~~C94A/C95A~~ C95A/C96A variant. In our assays, wild-type human ribonuclease inhibitor loses 50% of its activity at 0.007% volume per volume H₂O₂. By contrast, ~~C328A/C329A~~ C329A/C330A mutant human ribonuclease inhibitor retains 50% of its activity at 0.09% volume per volume H₂O₂. By this measure, the ~~C328A/C329A~~ C329A/C330A mutant form of ribonuclease inhibitor is ten to fifteen times more resistant to oxidative damage than is the wild-type human ribonuclease inhibitor. --

Page 14, delete the paragraph beginning on line 12, and substitute the following:

--The enhanced oxidation resistance of ~~C328A/C329A~~ C329A/C330A mutant hRI appears to result from the inhibition of the formation of a disulfide bond between the cysteines which would otherwise reside at residues ~~328 329~~ and ~~329 330~~. As shown in Figure ~~5 6~~, the individual ~~C328A~~ C329A and ~~C329A~~ C330A variants of mutant variants of hRI are just as resistant to oxidation by H₂O₂ as is the ~~C328A/C329A~~ C329A/C330A form of hRI. The simplest explanation for this result is that oxidation of the wild-type protein results in a ~~Cys-328-Cys-329~~ Cys-329-Cys-330 disulfide bond which cannot form in either of the single amino acid variants ~~C328A~~ C329A or ~~C329A~~ C330A, or in the double amino acid variant ~~C328A/C329A~~ C329A/C330A. --

Page 14, delete the paragraph beginning on line 33 (and continuing onto page 15), and substitute the following:

--Currently commercial human ribonuclease inhibitor is distributed in solutions containing millimolar levels of dithiothreitol (DTT). The presence of this reducing agent is included with the ribonuclease inhibitor to maintain the hRI in a reduced, and hence active, form. In many instances, such reducing agents are incompatible with laboratory protocols. Moreover, reducing agents are oxidized and thus rendered ineffective by the ubiquitous oxidant oxygen gas and transition metal ions. We find that replacing only one (i.e. ~~Cys328~~ Cys 329 or ~~Cys-329~~ Cys 330) of the 32 cysteine residues in hRI with an alanine residue substantially increases the

resistance of the molecule to oxidation, without compromising its affinity for RNAase A. This demonstrates that variants of hRI lacking a cysteine residue at positions ~~328~~ 329 or ~~329~~ 330, or the homologous positions in other ribonuclease inhibitors, will be more useful than wild-type ribonuclease inhibitors in many laboratory protocols. --